US ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY 40121

REPORT NO. 815

SERUM AGGLUTINATORS REACTING WITH PEPSIN TREATED Y-GLOBULIN:
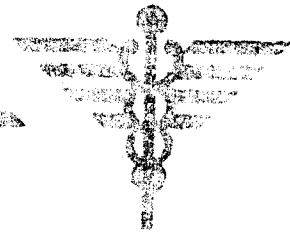I. "NATURALLY OCCURRING" REACTANTS IN THE SERUM OF SUBHUMAN PRIMATES

(Final Report)

by

Captain Stephen D. Litwin, MC
Lt Colonel Frank R. Camp, Jr., MSC
and
Lt Colonel Charles E. Shields, MC

7 March 1969

AD

SERUM AGGLUTINATORS REACTING WITH PEPSIN TREATED γ-GLOBULIN:
I. "NATURALLY OCCURRING" REACTANTS IN THE SERUM OF SUBHUMAN PRIMATES

(Final Report)

by

Captain Stephen D. Litwin, MC*
Lt Colonel Frank R. Camp, Jr., MSC
and
Lt Colonel Charles E. Shields, MC

Blood Transfusion Division
US ARMY MEDICAL RESEARCH LABORATORY
Fort Knox, Kentucky 40121

7 March 1969

*Present Address:
Department of Medicine, Section on Human Genetics,
Cornell University Medical College, New York, New York 10021

ABSTRACT

SERUM AGGLUTINATORS REACTING WITH PEPSIN TREATED γ-GLOBULIN:
I. "NATURALLY OCCURRING" REACTANTS IN THE SERUM OF SUBHUMAN PRIMATES

OBJECTIVE

To characterize serum agglutinators of infrahuman primates which react with pepsin digested immunoglobulin. Further, to compare these antibodies to similar reactants in human serum.

METHODS

Serological agglutination and inhibition of hemagglutination testing will be employed in addition to immunochemical techniques for characterization of antibody.

RESULTS AND CONCLUSIONS

The "pepsin agglutinators" of subhuman primates closely resembled similar reactants of human sera and offer a suitable experimental prototype for the study of autoimmune anti-globulin factors.

SERUM AGGLUTINATORS REACTING WITH PEPSIN TREATED γ-GLOBULIN: I. "NATURALLY OCCURRING" REACTANTS IN THE SERUM OF SUBHUMAN PRIMATES *

INTRODUCTION

Anti-globulins directed against pepsin revealed antigenic determinants are present in most human sera (1, 2). Of interest has been the demonstration that such "human pepsin agglutinators" (HPAs) autoreact with their own pepsin treated IgG (3). Despite considerable progress in establishing the specificity and immunochemical characteristics of these reactants (1-5), their etiology remains unclear.

Agglutinators, very similar to those described in humans, were encountered during studies of the serum of subhuman primates. These "subhuman pepsin agglutinators" (SPAs) were comparable in many respects to their human counterparts. It was felt that study of these antibodies would prove valuable in two ways: first, it would provide further information on comparative immune mechanisms among higher primates; and second. it would offer a suitable animal model for the examination of autoimmune mechanisms. This present report describes the incidence, specificity and immunochemical features of the SPAs.

MATERIALS AND METHODS

Erythrocytes: Whole human blood was drawn each week into ACD from a single donor and stored at 4°C.

Primate Sera: Normal human sera were obtained by single bleedings from healthy males, 19-25 years of age. The chimpanzee and orangutan serum samples were gifts of Dr. J. Moor-Jankowski of the Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center, New York. Bleedings from a colony of baboons and cynomologous and rhesus monkeys were obtained with the asisstance of Captain R. Bull and Major D. Hysell, US Army Medical Research Laboratory, Fort Knox. Kentucky, and stored frozen until use. With the exception of four of the chimpanzees, none of these animals had ever been immunized. Two owl monkey and shrew samples were obtained with the help of Major F. Ruymann, Walter Reed Army Institute of Research, Washington, D. C.

Immune Antisera: High titer human anti-Rh sera were obtained from individual donors through various commercial sources, including Spectra Biologicals, Inc., East Brunswick. New Jersey, and Dade Reagents, Miami, Florida. Antiserum Sp. 41 was generously provided by Dr. Erna van Loghem, Netherlands Red Cross, Amsterdam, The Netherlands. Dr. van Loghem had immunized a Macaca speciosa monkey with human O+ red blood cells. Anti-baboon antiserum was elicited in rabbits by injections of isolated baboon γ-globulin and subsequent absorption of the antiserum with human Fr. II. Specific anti-human gamma chain and mμ chain antisera were produced by immunizing rabbits with an IgG myeloma protein, and a Waldenstrom's macroglobulin, and then absorbing with pepsin treated Fr. II to remove light chain activity.

Glossary of abbreviations and terms: PA refers to a "pepsin agglutinator" in general, in either human or subhuman primate serum. "Pepsin agglutinators" are serum antibodies reacting with pepsin treated γ-globulin but not with whole γ-globulin. SPA refers to a subhuman serum "pepsin agglutinator." HPA refers to a human "pepsin agglutinator."

Isolation of γ-globulin: Starch block electrophoresis (6) was employed to isolate all primate γ-globulin. The protein profile of the slower electrophoretic regions was similar in man and the lower primates (7), and the cathodal two-thirds of the gamma region was eluted and used. The protein isolated from lower primates was considered to be immunoglobulin on the basis of its electrophoretic distribution and its cross-reactivity with anti-human gamma and mμ chain antisera (7, 8). Enzyme treatment, reduction and alkylation: pepsin (Pentex, Kankakee, Illinois) digestion was carried out at an enzyme to protein ratio of 2:100 in acetate buffer, pH 4.1, 0.1 M for 24 hours at 37°C (9). Digestion was stopped by dialysis against cold saline. Papain (Mann Research Labs., New York City) digestion was carried out at an enzyme protein ratio of 1:100 in the presence of .01 M cysteine and .002 M EDTA for 18 hours at 37°C (10). The papain Fab fragment was isolated by starch block electrophoresis and tested for contaminating Fc determinants by immunoelectrophoresis (11). Gamma globulin was reduced and alkylated as follows: the sample was first dialyzed against tris buffer 0.55 M, pH 8.2 for 2 hours; EDTA .002 M and 2 mercaptoethanol at either 0.1 or 0.2 M were added for 1 hour at room temperature and followed by iodoacetamide at a molarity 10% in excess of the mercaptoethanol. After an hour, the mixture was dialyzed against cold saline.

Immunological Methods: Ouchterlony agar diffusion (12), and microimmunoelectrophoresis (11) were performed as previously described. Euglobulin preparations were made from the primate serum by mixing 15 parts of distilled water with one part of the serum and permitting the mixture to stand at 5°C for 48 hours. The precipitate was washed twice in cold distilled water and reconstituted in tris-NaCl. Some of the "pepsin agglutinator" (PA) activity was always found in the precipitated euglobulin fraction. The euglobulin was applied to a G-200 Sephadex column (100 x 2.5 cm) equilibrated in 0.1 N tris, 0.5 M NaCl, pH 8.0. A flow rate of 9 ml/hr was used and 3.6 ml was collected per tube in the cold.

Starch Block Electrophoresis of Baboon Serum: A baboon serum was separated by starch block electrophoresis (6) and the protein was eluted from each 1 cm segment. After concentration, each eluate was tested for its SPA activity and for its ability to inhibit a SPA (vs. pepsin treated Ri coated cells).

Serologic Testing for Pepsin Agglutinators; Direct Agglutination and Inhibition Testing: A test tube (10 x 75 mm) three drop method (13) was employed in all serologic studies. Human O+ erythrocytes were sensitized in the following manner: one drop of packed red cells washed four times in normal saline was added to five drops of pepsin treated anti-Rh serum and four drops of normal saline. The reactants were incubated for 90 minutes at 37°C and washed four times in saline prior to use at approximately a 1% suspension.

Sp. 41 was a Macaca speciosa monkey serum which, after specific immunization, reacted with human O+ red blood cells. It was reduced with 0.2 M mercaptoethanol and alkylated to dissociate high molecular weight direct agglutinating antibody, and dialyzed. It was then pepsin treated and used to sensitize human red cells with M. speciosa γ-globulin in the same manner as human anti-Rh serum. Residual IgM monomeric units did not interfere with the sensitization for the pepsin revealed antigen, as judged by the effectiveness and specificity of this system in detecting PAs.

For direct agglutination testing, a drop of diluted test serum (usually the first dilution was 1:3) was added to one drop of sensitized red cells and one drop of saline. Samples were always tested against unsensitized red cells and, in some cases, absorption was necessary. The tubes were read for 1 to 4+ agglutination after 1 hour at room temperature and 60 seconds centrifugation (Serofuge, Clay-Adams). Inhibition testing was conducted according to the same procedure with the single difference being that instead of saline, a drop of inhibitor was used.

To establish that agglutinating activity was directed against a pepsin revealed site, the following steps were taken. A primate serum was absorbed with human red cells and titered against pepsin treated anti-Rh

2

sensitized red cells, starting at a serum dilution of 1:3. A dilution of serum was selected which resulted in a 2+ agglutination. This dilution was then blocked by inhibition testing with 1 mg/ml and 0.25 mg/ml of both whole Fr. II human γ-globulin and pepsin treated Fr. II human γ-globulin. If the pepsin Fr. II blocked the agglutination while the native Fr. II failed to, the serum was considered to contain a PA. This procedure was followed for most of the SPAs.

Pepsin treated human or subhuman γ-globulin or serum, when used as inhibitors of PAs, often had PA activity of their own against the detector red cells. Inhibitor controls, consisting of the inhibitor and the sensitized red cells, were always included in studies. It was difficult to avoid such PA activity in the inhibitor because of the wide occurrence of these agglutinators. As a rule, an effort was made to select proteins for inhibition studies which had low or absent antibody levels. In the occasional situation where selected proteins could not be used, such as testing for autospecificity, the inhibitors were employed at dilutions greater than the endpoint of their PA titers.

Gm Typing: This was performed as described previously (13), using known Gm typing systems (14).

Immunization of Cynomologous Monkeys with Bovine Serum Albumin: Four cynomologous monkeys received, intramuscularly, 5 mg of bovine serum albumin emulsified in equal volumes of Freunds' adjuvant, every month for 5 months. Bleedings were taken prior to immunization and 14 days after each injection. A final sample was withdrawn 8 weeks after the completion of the course of immunization. In two rabbits of the four, a precipitin curve was approximated by double agar diffusion, using the antisera taken after the third immunization. Immune precipitates, made at equivalence, were injected intravenously, instead of the fourth monthly intramuscular injection. Each sample was tested for precipitating anti-bovine serum albumin by double agar diffusion and for SPAs by means of three different pepsin treated coats, including Sp. 41.

RESULTS

Distribution and Frequency of SPAs Against Human Anti-Rh Coats: When pepsin digested human anti-Rh antibody coated on erythrocytes is used as the detector system, high frequencies of SPAs are present in chimpanzee, orangutan, baboon, and rhesus monkey serums. Table 1 summarizes the distribution of these factors. It is evident that the coats varied in their detection capacity with the most sensitive pepsin coats, such as Ri (15), finding a majority of sera positive at a dilution of 1:4. The number of serum specimens from the owl monkey (New World monkey) and the shrew (prosimian) are too few to comment on the distribution among these lower animals, but the single shrew sample with a SPA suggests that they can be found at early primate levels. Various other mammalian sera, such as dog, cat, rabbit and cow, were negative when studied with these detection systems.

Pattern of Reactivity of SPAs Against Different Human Anti-Rh Coats: The pattern of variability in the reactions of the subhuman primate sera with different human Rh coats was studied by two dimensional titration. The selected coats included several obtained from sera of phenotypes* Gm (z+a+g+f+b+), Gm (z+a+g+f-b-), and Gm (z-a-g-f+b+). The coats differed in their general level of sensitivity, but in addition to this, certain coats, such as DE and J, reacted unexpectedly well in certain instances with a baboon serum. In an effort to demonstrate separate populations of antibodies detected by single coats, baboon serum 27 was absorbed with pepsin anti-Rh sera Ri, H, and 83, and baboon serum 19 was absorbed with H, 83, and 71. When retested against the panel of pepsin coats, all agglutinating activity had disappeared. It was concluded

---

*Gm (z+a+g+f+b+) phenotype is considered heterozygous at the γ G1 and γ G3 Gm cistrons. Gm (z+a+g+f-b-) and Gm (z-a-g-f+b+) are the homozygotes for the Gm genes $Gm^{z,a}$, Gm^g and Gm^f, Gm^b, respectively (14).

that a pepsin revealed antigen was present to some degree on every Rh coat; that the pattern of variability could not be fully explained with the present data, but that it suggested antibody heterogeneity. The Gm constitution of the Rh coat did not appear to be of significance.

Detection of PAs with Macaca speciosa γ-globulin Coated Red Cells: SPAs were also detected when pepsin treated monkey γ-globulin was used as the antigen (Table 3). Sp. 41 serum coated effectively on human red cells, as determined by a high agglutination titer when sensitized cells were tested with a rabbit anti-baboon serum. Although the incidence of SPAs in Table 3 (M. spec. coat) cannot be compared to that in Table 1 (human Rh coat), since the degree of immunoglobulin sensitization might differ, it is significant that the trend of endpoint titers are opposite in direction to each other with different source γ-globulin coats; i.e., HPAs have higher titers than SPAs vs. human coats in contrast to the SPAs which have higher titers against the Sp. 41 coat than HPAs. This suggests that the PA activity might include multiple antibodies, some of which were species specific. The experiments described below provide further information on this point.

Specificity of SPAs: When a baboon and chimpanzee serum, each containing a SPA, are titrated against pepsin treated human Rh coat 71 and pepsin treated Sp. 41 coated cells, respectively, four agglutination systems are available. Each of these systems was tested for inhibition by human and baboon native γ-globulin, pepsin (F(ab')2, and papain Fab fragments, and the results outlined in Table 4. The SPA specificity was directed to the pepsin fragment, even when using the different species γ-globulin coats, and different species serum PAs. There was no inhibition by the papain Fab fragment in these experiments or in further studies. More complete data on the finer specifity of these antibodies for the subclasses of human IgG, and species cross-reactions will be presented in the future (15).

Cross Absorption Studies of PAs; A SPA Specific for Subhuman Primate γ-globulin: A single baboon serum and single human serum were selected for further study (Table 5). Both were absorbed with 3 mg/ml of pepsin treated baboon and human γ-globulin and then titered against pepsin human anti-Rh 71 coated cells and pepsin Sp. 41 coated cells. In most instances, all PA activity was removed. However, the absorption of baboon serum 22 with pepsin human γ-globulin, vs. Sp. 41 coated cells still left agglutinating activity. Reabsorption with pepsin treated human γ-globulin did not lower the titer. The "residual agglutinin" would appear to be SPA which is specific for subhuman pepsin treated γ-globulin. It is also apparent that a pepsin revealed antigen must be present on coat Sp. 41 but absent in human γ-globulin. The reverse of this situation, i.e., a PA residual activity specific for a human pepsin, revealed antigen was not detected.

The "residual agglutination" of the above baboon serum was further investigated by inhibition with different species of pepsin treated γ-globulin (Table 6). Native protein from baboon, monkey or man could not inhibit. Pepsin treated human γ-globulin failed to block at 1 mg/ml, while pepsin baboon γ-globulin, as well as pepsin chimpanzee and pepsin rhesus monkey γ-globulin, inhibited. The degree of inhibition was significantly different, as judged by inhibitory capacity (in inhibitory units).

Autoreactivity of SPAs: By inhibiting a SPA with its own pepsin digested serum*, the serum* of another member of the same species, and that of different species*, autoreactivity was demonstrated. In Table 7, two illustrative inhibition studies are outlined which indicate autoinhibition of a baboon and rhesus monkey SPA. In these studies, the inhibitors lost their blocking ability at 160- or 320-fold dilutions (not shown in Table 7), pointing out that the inhibitory capacity of autologous, isologous and heterologous γ-globulin was approximately the same. The above experiments were performed with a human pepsin Rh

---

*Similar results were obtained when pepsin treated γ-globulin was used.

4

coat as the detector antigen. Later studies of this type, employing the monkey Sp. 41 coat, demonstrated autoinhibition. All species possessing serum SPAs showed autoinhibition.

Immunologic Studies of SPAs; Column Chromatography: Five baboon sera, one rhesus monkey serum and one chimpanzee serum were converted to euglobulin preparations and subjected to G-200 column chromatography. Two of the chromatographic separations of baboon euglobulin are seen in Figure 1. Two peaks were obtained, the first one falling into the void volume. Protein cross-reacting with a rabbit anti-human $m\mu$ chain antiserum was restricted to peak one, while protein cross-reacting with a human gamma chain antiserum was restricted to peak two. It is believed that the void volume peak contained predominantly a higher molecular weight IgM type immunoglobulin, while the second peak had only lower molecular weight IgG type antibody. A large proportion of the IgG SPAs remained with the pseudoglobulin fraction and was not studied. Baboon 19 had the SPA activity restricted to the second peak, while baboon 27 had SPA associated with both peaks. A third baboon had activity in peak one only and the pseudoglobulin of this animal was PA negative. The remaining two baboons, the rhesus monkey and the chimpanzee euglobulin preparations had SPA activity in the second peak. The highest titers of agglutinator were coincident with the protein peaks. It seems that a majority of the SPAs are immunoglobulins related to human IgG, but that they also may be higher molecular weight IgM antibody. The eluates from under the first and second peak of baboon 27 were concentrated and studied by inhibition testing. The specificity of each isolated SPA preparation appeared no different than that of the same SPAs in whole serum.

Prozone Phenomena. Five baboon sera and five chimpanzee sera were reacted undiluted, 1:1, 1:2, 1:3, 1:4, 1:6, 1:7 and 1:10, against three different human pepsin treated coats and the pepsin Sp. 41 coat. There were no prozones.

Starch Block Electrophoretic Separation of a Baboon Serum: Whole serum of baboon 22 was separated by starch block electrophoresis. In Figure 2, the distribution of PAs is seen in relation to the protein concentration. A serum specific for baboon γ-globulin reacts with the protein in the gamma electrophoretic region. In the slowest electrophoretic zone, there is an inhibitor of the SPA, as judged by the ability of such eluates to block the agglutination between a strong SPA containing serum and pepsin coated Ri cells. These slow region eluates could also inhibit agglutination produced by baboon serum 22 (autoinhibition) (3).

Reduction with Mercaptoethanol: Williams and Lawrence reported the loss of the pepsin revealed antigen when IgG was treated with as low as .001 M mercaptoethanol (4). A selected group of baboon and chimpanzee sera was reduced, alkylated and retested. There was a partial loss of the pepsin revealed antigen when as little as .01 M mercaptoethanol was employed and complete loss of the antigen at 0.2 M.

Effect of Repeated Immunization on SPAs in Cynomologous Monkeys: Four cynomologous monkeys were repeatedly immunized with BSA in order to determine if chronic intensive immunization induced the formation of PAs or altered the level of existing antibodies. Three of the four animals had low or moderate levels of SPAs prior to immunization, while one was seronegative at the lowest dilution tested of 1:4. After the second immunization, the animals could be shown to have precipitating anti-BSA detectable by double agar diffusion. PAs were determined, using different human pepsin anti-Rh coats and Sp. 41 coat. There was no significant change from the sample taken on day zero to the sample taken 8 weeks after the injections had stopped. The seronegative animal developed a SPA at 1:6, but this was not considered significant.

DISCUSSION

Human pepsin agglutinators have been carefully studied and can be well characterized. In the original description of the HPAs in 1963, Osterland, Harboe, and Kunkel (1) pointed out that the antibody was

5

usually a 7S protein reacting with a hidden antigen on the pepsin F(ab')2 fragment, and that it differed from "classical rheumatoid factor." Williams and Lawrence (4) noted that the HPAs reacted with pepsin digested IgG myeloma proteins, and that the determinant was progressively lost with mercaptoethanol reduction. In further work, it was shown that human sera containing pepsin agglutinators often possessed low molecular weight substances of slow electrophoretic mobility which could inhibit the agglutination reaction (3). In a series of studies, Natvig (5) points out considerable heterogeneity within the human serum pepsin agglutinators and found, in some cases, a relationship to the Gm factors.

These present results provide strong evidence for the presence in many subhuman primate sera of anti-globulins reacting with an enzyme revealed antigenic determinant. These agglutinators are recognized by reacting a test serum with detector particles of red cells coated with pepsin digested γ-globulin. The specificity of the agglutination is confirmed and further delineated by blocking a SPA with immunoglobulin fragments. For the purposes of these studies, a serum was defined as positive for PAs if it reacted directly and was inhibited by pepsin treated but not whole γ-globulin. By these criteria, similar reactants are present among many different primates. When representative SPAs were selected for intensive study, they appeared to possess the properties of immunoglobulins on G-200 chromatography and electrophoresis. The SPA antibodies were widely dispersed electrophoretically over the gamma region except in the slowest zone (Fig. 2). In addition, there is evidence suggesting heterogeneity of antibody in the variable pattern of reactions with different pepsin Rh coats (Table 2), and the presence of antibody activity in both high and low molecular weight immunoglobulin peaks after G-200 chromatography.

In most respects, the SPAs of these studies had features very similar to those noted above for their counterparts in human serum. Both SPAs and HPAs have high frequencies in normal primates (2); both have specificity for pepsin altered γ-globulin but fail to react with whole protein (1); both exhibit autoreactivity (3); both are of unknown etiology and appear uninfluenced by exogenous immunization (2); and, finally, both are often IgG but may be IgM or both (1, 16). The close resemblance between human and lower primate PAs in their immunochemical features and in their heterogeneity is strengthened by an extensive cross-reactivity. Pepsin agglutinators detect antigens present on γ-globulin of species other than their own, as was immediately apparent during the early phases of this study when the SPAs were first noted utilizing human pepsin coats. Both SPAs and HPAs, when reacted with either a human or a monkey pepsin altered γ-globulin, retain the same specificity (Table 4). The present evidence supports close parallelism of PA antibody reactants at different phylogenetic levels.

Notwithstanding the similarities between HPAs and SPAs, there is evidence of heterogeneity within the agglutinators in the form of species reactive antibodies. This was suggested by the stronger agglutinability of SPAs against monkey γ-globulin on the one hand, and that of the HPAs with human γ-globulin on the other. This postulate was supported by the finding of a SPA which, after suitable absorption, would react only with lower primate pepsin γ-globulin. This data indicated a minimum of two discrete PA antibody populations, one of which doesn't combine with human γ-globulin. The above absorbed SPA (specific for infrahuman pepsin γ-globulin) still is inhibited by several species of subhuman pepsin γ-globulin, but to substantially different degrees; a fact which suggests that the absorbed SPA may contain further subspecies. The inability to demonstrate the reverse phenomenon, i.e., an absorbed HPA reacting only with human pepsin γ-globulin, probably reflects the limited number of samples tested.

Aside from man, there are reports of anti-globulins in rabbit sera against enzyme revealed γ-globulin determinants, by Mandy and co-workers (17). "Homoreactant" is a serum factor found in all rabbit sera which agglutinates red cells sensitized by rabbit papain Fab fragments. Recent studies (18) indicate that there are different "homoreactants" with specificity for the Fab' and F(ab')2 fragments, as well as the Fab. Further evidence of buried antigenic sites in rabbit γ-globulin which are related to human antigens, can be derived from the studies of Williams and Kunkel (19), in which it was shown that serum from rabbits

6

immunized with autologous papain digested γ-globulin precipitated both native rabbit and human gamma globulin. "Homoreactant" of rabbits and the SPAs and HPAs of primates constitute a class of serum reactants within which certain features are consistent. The striking characteristics of this group of serum antibodies are: they are present in many if not all normal sera from that species; they autoreact; they give evidence of antibody heterogeneity. It is intriguing to consider what functions such reactants might perform, perhaps in the complexing and rapid excretion of catabolized IgG, or of immune complexes.

Macroglobulin antibodies to light chain determinants, blocked in whole γ-globulin, have been detected by Schoenfeld and Epstein (20) in a number of rheumatoid human sera. They are predominantly of a different molecular size but, in certain respects, resemble the other serum reactants discussed.

Closely connected to the possible physiological role of PAs is the problem of their etiology. The wide distribution and high incidence of SPAs among lower primates, in conjunction with similar data noted in man, suggests that these antibodies arise from normally operative immune mechanisms, rather than pathologic derangements. It is difficult to conceive of any pathogenic effects resulting directly from a virtually universal serum factor. Directly pertinent are recent findings by Waller (21) of agglutinators in most human serum against anti-Rh antibodies digested with four different proteolytic enzymes; pepsin, papain, ficin and bromelin. The agglutinators appeared specific for each enzyme modification except for cross-reactions between the papain and ficin agglutinators.

One widely suggested cause of PAs has been immunization. In support of this theory has been the demonstration that HPAs may combine with immune complexes in vitro (16). Waller (2) immunized 33 humans with Vi or Brucella antigens and observed no alteration of PA levels. Similar observations have been made in a group of young Army recruits receiving repeated vaccinations during an 8-week basic training period (22). Despite the ten closely spaced immunizations, the subjects did not show any significant rise or fall in their levels of HPAs. During the present studies, cynomologous monkeys were given monthly injections of BSA emulsified in complete Freunds' adjuvant. The use of complete adjuvant and the chronicity of the stimulation, it was hoped, would provide an experimental regimen more rigorous than encountered in man. Titrations of SPAs prior to, during and after the immunizations showed no significant changes. It should be noted that three of the four monkeys possessed SPAs prior to the initiation of the studies so that any proper stimulus should elicit a recall response. It is evident that the character of the elicited immune response may have been unsuitable. Christian (23) and co-workers have pointed out a correlation between the presence of nonprecipitating antibodies and the subsequent development of chronic nephritis in animals.

The present studies on the characterization of SPA indicate that they closely parallel similar human autoantibodies. Thus, the higher primates may serve as suitable laboratory prototypes for the study of autoimmune mechanisms. The biological significance of PAs is of current importance to workers in the fields of autoimmunity, γ-globulin catabolism and structure, and comparative immunology.

SUMMARY

Anti-globulins directed against both human and subhuman primate pepsin digested γ-globulin were detected in subhuman primate sera. The specificity of the SPAs, their wide distribution in normal animal sera, many of their immunochemical features, and their autoreactivity all paralleled the properties of HPAs. It was concluded that the PAs at different primate levels were closely related to one another.

The majority of SPAs cross-reacted with pepsin treated human γ-globulin. However, a subspecificity was demonstrated in a SPA which reacted selectively with only infrahuman proteins. In addition, there was other evidence for heterogeneity of the SPA antibodies.

7

Attempts to induce or boost SPA levels by deliberate immunization of cynomologous monkeys with bovine serum albumin were unsuccessful.

LITERATURE CITED

1. Osterland, C. K., M. Harboe and H. G. Kunkel. Vox Sang. 8: 133, 1966.

2. Waller, M. and K. Blaylock. J. Immunol. 97: 438, 1966.

3. Lawrence, T. G., Jr. and R. C. Williams, Jr. J. Exper. Med. 125: 233, 1967.

4. Williams, R. C., Jr. and T. G. Lawrence, Jr. J. Clin. Invest. 45: 714: 1966.

5. Natvig, J. B. Acta Path. et Microbiol. Scandinav. 66: 369, 1966.

6. Kunkel, H. G. Methods Biochem. Anal. 1: 141, 1954.

7. Picard, J., J. Heremans and G. Vanderbroek. Vox Sang. 7: 190 and 425, 1962.

8. Poulik, M. D. Primates in Med. 1: 68, 1968.

9. Nisonoff, A., G. Markus, and F. C. Wissler. Nature, 189: 293, 1961.

10. Porter, R. R. Biochem. J. 73: 119, 1959.

11. Scheidegger, J. J. Intern. Arch. Allergy and Appl. Immunol. 7: 102, 1955.

12. Ouchterlony, O. Acta Path. et Microbiol. Scandinav. 32: 231, 1953.

13. Harboe, M., C. K. Osterland, M. Mannik and H. G. Kunkel. J. Exper. Med. 116: 719, 1962.

14. Litwin, S. D. and H. G. Kunkel. J. Exper. Med. 125: 847, 1967.

15. Litwin, S. D. In preparation.

16. Harboe, M., B. Rau and K. Aho. J. Exper. Med. 121: 502, 1965.

17. Mandy, W. J., H. H. Fudenberg and F. B. Lewis. J. Immunol. 95: 501, 1966.

18. Mandy, W. J. J. Immunol. 99: 815, 1967.

19. Williams, R. C. and H. G. Kunkel. Proc. Soc. Exper. Biol. and Med. 112: 554, 1963.

20. Schoenfeld, L. S. and W. V. Epstein. Vox Sang. 10: 482, 1965.

21. Waller, M. Vox Sang. 13: 233, 1907.

22. Litwin, S. D. and C. E. Shields. SAMRL Report No. 804, Ft Knox, Ky, 1968.

23. Pincus, T., R. Haberkern and C. L. Christian. J. Exper. Med. 127: 819, 1968.

8

TABLE 1

DISTRIBUTION AMONG PRIMATES OF AGGLUTINATORS AGAINST HUMAN PEPSIN TREATED ANTI-Rh COATS

SPECIES*	NO. TESTED	NUMBER SAMPLES POSITIVE[+] PEPSIN ANTI-Rh COAT					RECIPROCAL OF TITER RANGE PEPSIN COAT Rı
		Rı	71	OB	J	H	
Man	25	23	17	4	13	8	24-48
Chimpanzee	21	12	11	0	3	NT	8-16
Orangutan	5	3	2	0	0	NT	8-16
Baboon	28	20	17	4	6	4	8-16
Rhesus Monkey	7	3	0	0	0	0	4-8
Owl Monkey	2	0	0	NT	NT	NT	—
Shrew	1	1	0	NT	NT	NT	8

*The chimpanzee and orangutan are representatives of the great apes (Pongo); the baboon and rhesus monkey the Old Wor... nonkeys (Cercopithecoidea); the owl monkey the New World monkeys (Ceboidea); and the shrew is a Prosimia.

+Positive at a dilution of 1:4.

NT - Not tested.

TABLE 2

PATTERN OF REACTIVITY OF FIVE BABOON SERA AGAINST TEN DIFFERENT HUMAN PEPSIN TREATED ANTI-Rh COATS

PEPSIN TREATED ANTI-Rh COAT

RECIPROCAL OF TITER

BABOON SERUM	Rı	J	DE	H	71	07	55	83	54	51
19	12	12	0	6	24	12	12	3	12	24
23	24	0	6	0	12	3	6	12	12	12
27	12	0	24	3	0	0	0	0	0	0
22	6	6	0	3	6	6	6	6	6	12
34	24	24	3	3	24	12	12	24	24	48

9

TABLE 3

PRIMATE SERUM AGGLUTINATORS REACTING WITH
A PEPSIN TREATED MACACA SPECIOSA γ-GLOBULIN COAT

	NO. TESTED	NUMBER SAMPLES POSITIVE* PEPSIN TREATED M. SPEC. 41 COAT	RECIPROCAL OF TITER RANGE PEPSIN M. SPEC. 41 COAT
Man	12	5	4 - 8
Chimpanzee	10	5	8 - 16
Orangutan	2	1	48
Baboon	18	14	24 - 48
Rhesus Monkey	4	4	24 - 48
Owl Monkey	1	0	—
Shrew	1	1	48

*At a dilution of 1:4 or higher.

10

TABLE 4

SPECIFICITY OF SUBHUMAN PEPSIN AGGLUTINATORS
DETERMINED BY INHIBITION TESTING

SPA	ERYTHROCYTE γ · GLOBULIN COAT	INHIBITOR* PROTEIN	of	Inhibition Testing INHIBITORY UNITS** MAN	BABOON
Baboon 31 Serum	Human Pepsin Anti-Rh 71	γ·Globulin		0	0
		Pepsin γ-Glob.		6	6
		Papain γ-Glob.		0	0
Baboon 31 Serum	M. Spec. 41 Pepsin Treated	γ-Globulin		0	0
		Pepsin γ-Glob.		4	6
		Papain γ-Glob.		1	0
Chimpanzee Be Serum	Human Pepsin Anti-Rh 71	γ·Globulin		0	0
		Pepsin γ-Glob.		5	4
		Papain γ-Glob.		0	0
Chimpanzee Be Serum	M. Spec. 41 Pepsin Treated	γ·Globulin		0	0
		Pepsin γ-Glob.		5	6
		Papain γ-Glob.		0	0

* γ-globulin refers to intact untreated γ-globulin isolated from a starch block; pepsin γ-globulin is the F(ab')2 fragment and papain γ-globulin the Fab fragment of the same γ-globulin.

**Inhibitory units are the number of serial twofold dilutions of inhibitor γ-globulin, starting at 1 mg/ml, which completely inhibit agglutination.

11

TABLE 5

CROSS ABSORPTION OF PEPSIN AGGLUTINATORS

PEPSIN TREATED HUMAN
ANTI-Rh COAT 7170

PEPSIN TREATED M. SPEC.
41 γ-GLOBULIN COAT

RECIPROCAL OF TITER OF SERUM PA
AFTER ABSORPTION* WITH

PA	UNABS	PEPSIN BABOON	PEPSIN HUMAN	UNABS	PEPSIN BABOON	PEPSIN HUMAN
Baboon 22 Serum	40	0	0	80-160	0	8
Human 11 Serum	20	0	0	10	0	0

*The absorbents were pepsin treated γ-globulin from baboon or man.

12

TABLE 6

A PEPSIN AGGLUTINATOR SPECIFIC FOR SUBHUMAN PRIMATE γ-GLOBULIN: SERUM BABOON 22 ABSORBED WITH HUMAN F(ab')2 VS. PEPSIN M. SPEC. γ-GLOBULIN COATED CELLS

Inhibitor F(ab')2[*]

SPECIES	INHIBITORY UNITS[+]
Man	0
Chimpanzee	3
Gibbon	2
Baboon	6
Rhesus Monkey	4

[*]Whole γ-globulin or serum of any primate failed to inhibit.

[+]Inhibitory units are the number of serial twofold dilutions of inhibitor.

γ-globulin, starting at 1 mg/ml, which completely inhibit agglutination.

The agglutinator was baboon serum 22, diluted 18-fold, and reacted with M. spec. γ-globulin.

TABLE 7

AUTOSPECIFICITY OF SUBHUMAN PEPSIN AGGLUTINATORS
DETERMINED BY INHIBITION TESTING

AGGLUTINATOR	INHIBITOR	TEST* RESULTS AGGLUTINATION		
		RECIPROCAL OF DILUTION OF INHIBITOR		
SPA IN SERUM	PEPSIN TREATED SERUM OF	20	40	80
Baboon 19	Saline	2+	2+	2+
	Baboon 19	0	0	0
	Rhesus Monkey 2	0	0	0±
	Baboon 3	–	–	–
Rhesus Monkey	Saline	2+	2+	2+
	Baboon 19	0	0	0±
	Rhesus Monkey 2	0	0	0
	Rhesus Monkey 1	0	0	0

*Human anti-D coat Ri was used for testing: the results were similar when coat M. Spec. 41 was employed. Agglutination 1-4+ indicates no inhibition whereas an O reaction indicates inhibition.
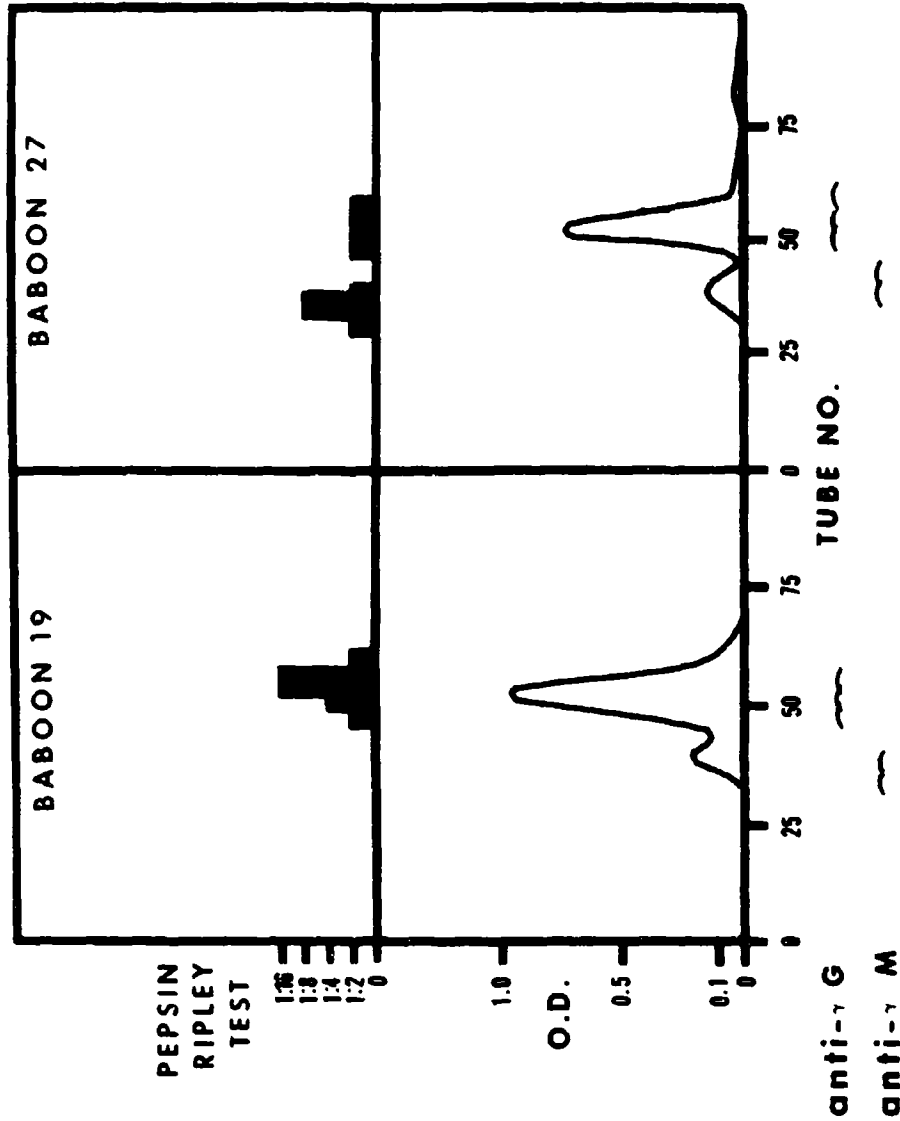
Fig. 1. G-200 column chromatography of euglobulin preparations from two baboon sera. The upper portion of the figure indicates the SPA activity vs. pepsin treated Ri coated red cells. Below the protein scans of the two peaks are shown precipitin arcs seen on double agar diffusion plates using specific anti-human gamma and mμ chain antisera.
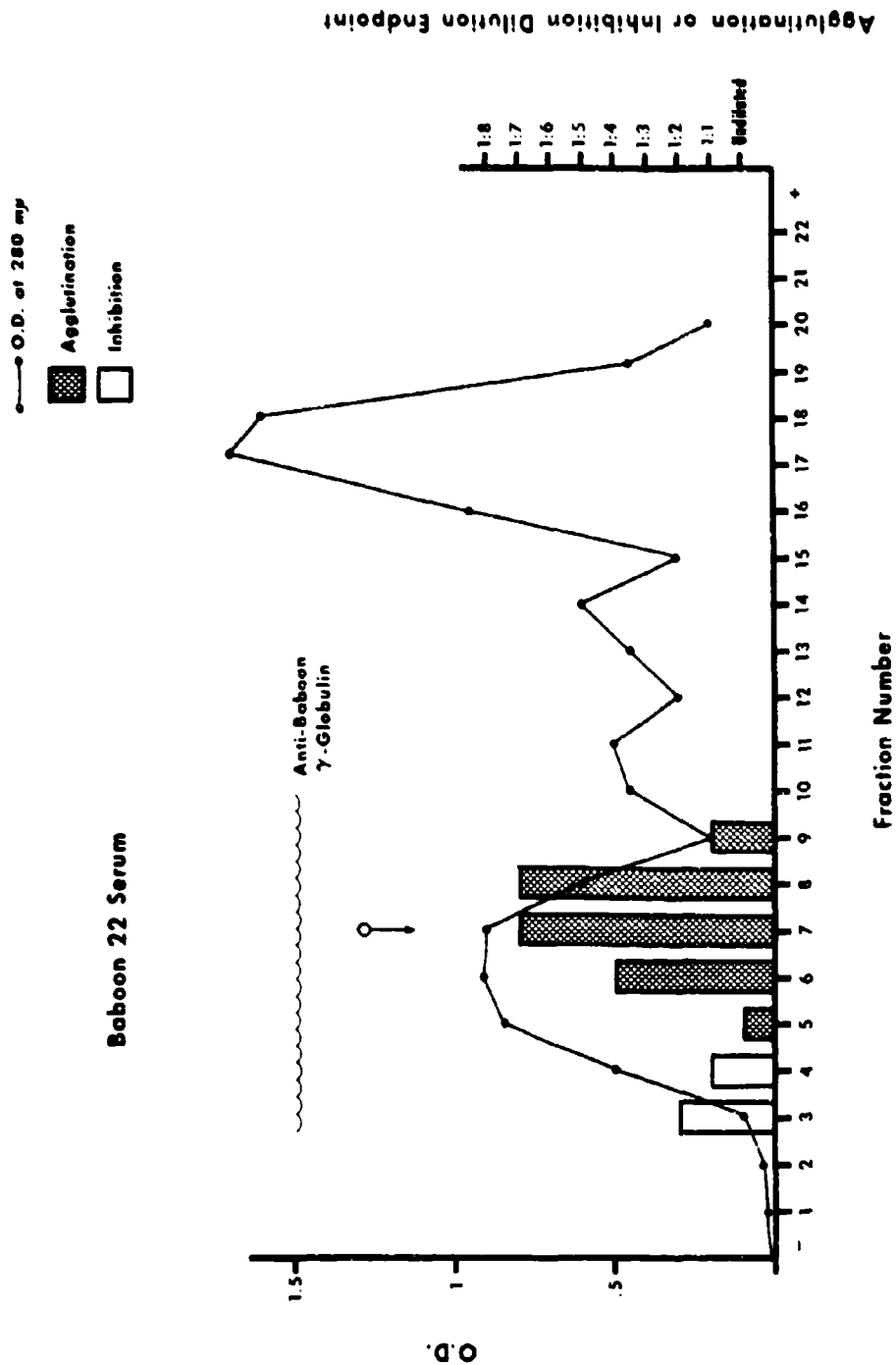
Fig. 2. Starch block zone electrophoresis separation of baboon serum 22 showing distribution of SPA activity against pepsin Ri coated red cells. The dark solid bars indicate the PA activity of the eluate and the open bars inhibitory capacity of the eluate for a SPA (baboon serum 19 vs. pepsin Ri coat). The precipitin reaction of the eluate, when tested by double agar diffusion with anti-baboon γ-globulin antiserum, is shown by the arcs.

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
US Army Medical Research Laboratory Fort Knox, Kentucky 40121	UNCLASSIFIED
	2b. GROUP

3. REPORT TITLE

SERUM AGGLUTINATORS REACTING WITH PEPSIN TREATED γ-GLOBULIN: I. "NATURALLY OCCURRING" REACTANTS IN THE SERUM OF SUBHUMAN PRIMATES

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Final Report

5. AUTHOR(S) (First name, middle initial, last name)

CPT Stephen D. Litwin, MC, LTC Frank R. Camp, Jr., MSC, and LTC Charles E. Shields, MC

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
7 March 1969	16	23

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 3A062110A821	815
c. Task No. 00	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d. Work Unit No. 155	

10. DISTRIBUTION STATEMENT

This document has been approved for public release and sale; its distribution is unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	US Army Medical Research and Development Command Washington, D. C. 20315

13. ABSTRACT

The sera of subhuman primates were found to contain anti-gamma globulins directed against pepsin revealed antigens of primate immunoglobulin. These reactants were compared to similar antibodies of human serum and showed striking similarities in specificity and immunochemical characteristics. Further, both human and subhuman "pepsin agglutinators" were widely distributed and "autoreactive." (U)

Attempts to induce formation of alteration of the titer of these antibodies in monkeys by hyperimmmunization were unsuccessful. (U)

DD FORM **1473** REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Subhuman Primates						
Pepsin Agglutinators						
Hyperimmunization						